

Remarks

At the time of the instant Office Action, claims 1-28, 30-34 and 36 were pending in the subject application; claims 1-6, 12-23, and 34 were withdrawn; and claims 7-11, 24-28, 30-33 and 36 were considered. Applicant notes with appreciation the Examiner's acknowledgment to the effective filing date of this application as being July 19, 2004.¹ Applicant notes further with appreciation the withdrawal of the previously issued rejection under the first paragraph of 35 U.S.C. § 112 with respect to the encapsulating layer.² By this Amendment, claim 24 has been amended to provide greater clarity. No new matter is introduced by these claim amendments. Favorable reconsideration of pending claims 7-11, 24-28, 30-33 and 36 is respectfully requested.

Remarks Directed to Claim Rejections under 35 U.S.C. § 101

The claimed invention stands rejected under 35 U.S.C. § 101 for the same reasons set forth in the record.³ Applicant's remarks previously submitted⁴ are not reproduced herein. However, Applicant, through his counsel, wishes to provide the following comments on the Examiner's "Response to Arguments".⁵

In the "Response to Arguments," the Examiner continues to question the usefulness of the claimed invention as the medically efficacious substance is not released and continues to argue that a pharmaceutical must be released for action in a conventional sense. Applicant respectfully traverses. A composition, or particularly a preparation for used as a medicament as recited in the claims, does not need to be a pharmaceutical to be useful. In fact,

¹ See page 2 of the instant Office Action.

² See page 2 of the instant Office Action.

³ See page 3 of the instant Office Action and page 3 of the Office Action dated January 7, 2009.

⁴ See pages 8-12 of the Amendment dated April 7, 2009.

⁵ See pages 3-5 of the instant Office Action.

the claimed preparation is neither recited nor intended to function as a pharmaceutical in the conventional sense the Examiner has suggested. The claimed preparation is simply used as a medicament, or a material or substance used in therapy. The type of use and the area of application for the claimed preparation may not be very conventional, but that alone does not negate its usefulness pursuant to the utility requirement pursuant to 35 U.S.C. 101.

In the "Response to Arguments," the Examiner opines that Applicant's remarks with respect to a proposed mechanism of action are not persuasive because the Examiner can find no evidence to that effect presented by the Applicant. The Examiner further presents page 158 of *Rastogi in Cell and Molecular Biology* (hereinafter "*Rastogi*") in an attempt to disqualify Applicant's proposed mechanism of action as not within the teaching of *Rastogi*.⁶ Notably *Rastogi* is a fifteen (\$15) dollar elementary level paperback book, with its cover copy shown in Exhibit I. *Rastogi* allocates no more than 10 pages in an attempt to cover the whole complex field of signal transduction. Applicant respectfully submits, as Exhibit II, relevant portions of *Medical Physiology* by WF Boron and EL Boulpaep published by Elsevier Saunders in 2003 and updated in 2005 at Chapter 4 "Signal Transduction" page 87:

The evolution of multicellular organisms necessitated the development of mechanisms to tightly coordinate the activities among cells. Such communication is fundamental to all biological processes, ranging from the induction of embryonic development to the integration of physiological responses in the face of environmental challenges.

Classically, neural and endocrine tissues have been considered the major signaling systems. However, as our understanding of cellular and molecular physiology has increased, it has become evidence that all cells can receive and process information. *External signals*, such as odorants, chemicals that reflect metabolic status, ions, hormones, growth factors, and neurotransmitters can all serve as chemical messengers linking neighboring or distant cells.

Clearly, the known science corresponds very well with the paragraphs [0065] to [0068] of the published specification and Applicant should be commended for his unique insight into the science, resulting in a practical and useful product to better human and animal health.

⁶ See page 4 of the instant Office Action.

Therefore, the original specification provides a sound explanation as to one working mechanism of operation, which finds sufficient support in the known science.

Applicant respectfully traverses the Examiner's assertion that no signaling could take place when the medically efficacious material of the claimed preparation is not released. Cell signaling does not necessarily require the involvement of a hormone or a neuronal transmitter. As stated elsewhere in this Amendment, the medically efficacious substance of the claimed preparation can induce cell signaling response by means of, for instance, the molecules of water vapor, a gas, which are permeable through the gas-permeable coating layer, and thus can be made adjacent to ions of the medically efficacious substance.

At the very least, the Patent Office is not a functional equivalent to the Food and Drug Administration (FDA). Just like the FDA's role is not to analyze patent claims, the Patent Office's role is not to study the medical feasibility or efficacy of a claimed invention. In fact, as Applicant has raised previously⁷, the Patent Office is required to rely on the inventor's understanding of his invention and should focus on and be receptive to assertions made by the Applicant that an invention is useful⁸ in considering whether an application is "useful" under 35 U.S.C. § 101.

The utility of Equiwinner patches has been clearly set out. Applicant in his Declaration dated April 3, 2009 states that the cup discs were filled with sodium chloride sealed with a matrix of white beeswax, the wax being hardened with talc and cornstarch⁹ and the Equiwinner patches have been sold to professional horse trainers in various countries.¹⁰ Thus, the Equiwinner patches clearly evidence the utility of the claimed invention. Equiwinner patches are

⁷ See page 8 of the Amendment dated April 7, 2009.

⁸ See for instance, MPEP 2107.01(1).

⁹ See paragraph 16.

¹⁰ See paragraph 17.

effective for correcting a number of conditions in horses¹¹ and their success is described in more detailed in Applicant's Declaration.¹² The Examiner is asked to provide evidence to support his assertion that the Equiwinner patches are not effective.

The Examiner states that¹³ the information provided in the examples does not meet the currently accepted scientific standards for determining the efficacy of new pharmaceutical compositions and that the currently accepted practice uses double blind controls in which one group receives the new drug and a control group receives a placebo. In response, Applicant respectfully submits Exhibit III, which provides a list of granted U.S. Patents disclosing new drug entities, however none of which involves any use of placebo controls in the examples given.

For at least the reasons set forth above, reconsideration and withdrawal of the rejections is solicited.

***Remarks Directed to Claim
Rejections under 35 U.S.C. § 112***

Claims 7-11, 24-28, 30-33 and 35-36 stand rejected under 35 U.S.C. § 112, first paragraph, for failing the enablement requirement for similar reasons set forth previously in the record.¹⁴

Applicant's previously submitted remarks are not reproduced herein. However, Applicant, through his counsel, wishes to comment on the Examiner's "Response to Arguments."¹⁵

¹¹ See paragraph 14.

¹² See paragraph 18.

¹³ See page 7 of the instant Office Action.

¹⁴ See pages 5-8 of the instant Office Action and see also pages 4-5 of the Office Action dated January 9, 2009.

¹⁵ See pages 8-9 of the instant Office Action.

The Examiner continues to opine that Applicant's claimed invention lacks enablement as there lacks evidence to show a drug not released is effective in producing any medical effect.¹⁶ Applicant respectfully traverses for at least the reasons set forth previously on the record.¹⁷

With respect to the 2003 Nobel lecture of MacKinnon previously submitted, the Examiner opines that the pattern of water molecules around the instantly claimed preparation would not be expected to resemble the pattern of water molecules around an ion such as sodium or potassium as described in the lecture.¹⁸ Notably, and contrary to the Examiner's asserted characterization, no "pattern of water molecules around the instantly claimed preparation" has ever been mentioned in the Applicant's previously submitted Amendment dated April 7, 2009. Page 18 of the Amendment dated April 7, 2009 specifies that Applicant's claimed invention in at least one embodiment allows for sodium chloride and other medically efficacious substances to be placed in close proximity to body cells, and hence ion channels on the body cells.

According to certain instances of the claimed invention, the medically efficacious substances such as ions are within the encapsulation but surrounded by water vapor, wherein the water vapor can be attracted in through the liquid impermeable but gas permeable layer as stated previously.¹⁹ When the claimed preparation is configured as a tablet, the tablet can be surrounded by liquid water when taken into one's digestive track. Thus, there is a continuous thread of water between the ions in the tablet and the cells from the body. MacKinnon makes clear that the cells recognize the pattern of molecules around the ions²⁰ and this recognition induces the opening of the ion channels and hence the formation of electrical signals. Thus, and contrary to the

¹⁶ See page 5 of the instant Office Action.

¹⁷ See pages 17-19 of Applicant's Amendment dated April 7, 2009.

¹⁸ See pages 8-9 of the instant Office Action.

¹⁹ See for instance Exhibit 4 of Applicant's Amendment dated April 7, 2009.

²⁰ Note that the cells recognize the pattern of molecules around the ions, but not around the preparation as a whole, as the Examiner has suggested on page 9 of the instant Office Action.

Examiner's assertion,²¹ the ions do not need to be released for the cell signaling to take place and it is sufficient that the ions are adjacent to, for instance, water vapor, which can enter the liquid impermeable but gas permeable layer.

Similar reasoning can be applied to the use of the medically efficacious substance according one or more embodiments of the present invention. The Examiner has mentioned aspirin, presumably because aspirin is known to be insoluble, or barely soluble in water. However, aspirin is first hydrolyzed to salicylic acid, as shown in the Example 3 of the specification. Salicylic acid is capable of dissolving in water and therefore has ionic properties. Please see for instance the 2002 review "Anti-inflammatory effects of aspirin and sodium salicylate" by Amman R and Peskar BA, currently submitted as Exhibit IV, wherein the signaling effect of salicylate is explored, *see* for instance the Abstract at line 11 and section 5, paragraph 2 at lines 1-3.

For at least the reasons set forth above, Applicant respectfully requests reconsideration and withdrawal of these rejections.

***Remarks Directed to New Claim
Rejections under 35 U.S.C. § 112***

Claim 24 is rejected under 35 U.S.C. § 112, first paragraph, for the recitation of the term "substantially."²² In addition, claims 7-11, 24-28, 30-33, and 35-36 are newly rejected under 35 U.S.C. § 112, second paragraph, due to the recitation of "substantially" in claim 24. *Id.*

The term "substantially" is deleted from claim 24 to advance prosecution of this application. Reconsideration and withdrawal of the rejections is solicited.

²¹ See for instance page 9 of the instant Office Action.

²² See pages 9-11 of the Office Action.

CONCLUSION

Applicant submits that the claims are now in condition for allowance, and respectfully requests a Notice to that effect. If the Examiner believes that further discussion will advance the prosecution of the application, the Examiner is highly encouraged to telephone Applicant's attorney at the number given below.

Please charge any fees or credit any overpayments as a result of the filing of this paper to our Deposit Account No. 02-3978.

Respectfully submitted,

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Date: September 23, 2009

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EXHIBIT I

CELL AND MOLECULAR BIOLOGY

Second Edition

Cell and Molecular Biology, Second Edition gives an extensive coverage of the fundamentals of molecular biology; the problems it addresses and the methods it uses. Molecular biology is presented as an information science, describing molecular steps that nature uses to replicate and repair DNA; regulate expression of genes; process and translate the coded information in mRNA; modify and target proteins in the cell; integrate and regulate metabolism.

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- An introductory chapter on bioinformatics included to emphasise that molecular processes can be addressed computationally.
- Extensive glossary.

S.C. Rastogi, former Professor of Biological Sciences at Birla Institute of Technology and Science, Pilani, has a vast and variegated experience of teaching and research in the fields of molecular biology, biochemistry and enzyme biotechnology. A biologist of repute and accredited with many an educational innovation, he has been a constant participant in the course development activity at BITS and other universities. He is a renowned author of several books

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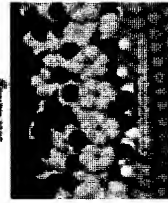
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S.C. Rastogi



Cell Biology

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Pilani, Rajasthan.

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Binding : Paperback



PDF SAMPLE CHAPTER

About the Book:

Especially designed for students and professionals, this textbook presents fundamentals of cell biology, from microscopic to the molecular level, in a relevant and user-friendly manner, supported by excellent diagrams, micrographs and tables.

EXHIBIT II

MEDICAL PHYSIOLOGY

A Cellular and Molecular Approach

UPDATED-EDITION

Walter F. Boron, MD, PhD

Professor

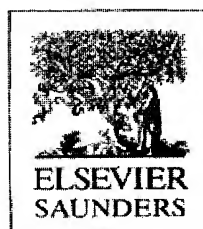
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MEDICAL PHYSIOLOGY

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Signal Transduction

Laura M. Roman

The evolution of multicellular organisms necessitated the development of mechanisms to tightly coordinate the activities among cells. Such **communication** is fundamental to all biological processes, ranging from the induction of embryonic development to the integration of physiological responses in the face of environmental challenges.

Classically, neural and endocrine tissues have been considered the major signaling systems. However, as our understanding of cellular and molecular physiology has increased, it has become evident that all cells can receive and process information. External **signals** such as odorants, chemicals that reflect metabolic status, ions, hormones, growth factors, and neurotransmitters can all serve as **chemical messengers** linking neighboring or distant cells. Even external signals that are not considered chemical in nature (e.g., light and mechanical or thermal stimuli) may ultimately be transduced into a chemical messenger. Most chemical messengers interact with specific **cell-surface receptors** and trigger a cascade of secondary events, including the mobilization of diffusible intracellular **second-messenger systems** that mediate the cell's response to that stimulus. However, hydrophobic messengers, such as steroid hormones and some vitamins, can diffuse across the plasma membrane and interact with **cytosolic or nuclear receptors**. It is now clear that cells use a number of different, often intersecting, intracellular signaling pathways to ensure that the cell's response to a stimulus is tightly controlled.

MECHANISMS OF CELLULAR COMMUNICATION

How Cells Communicate with Each Other by Direct Contact Through Gap Junctions

Neighboring cells can be electrically and metabolically coupled by means of **gap junctions** formed between apposing cell membranes (p. 164). These water-filled channels facilitate the passage of inorganic ions and small molecules, such as Ca^{2+} and 3',5'-cyclic adenosine monophosphate (cAMP), from the cytoplasm of one cell into the cytoplasm of an adjacent cell. Mammalian gap junctions permit the passage of molecules that are less than approximately 1200 Da but restrict the movement of molecules that are greater than approximately 2000 Da. Gap junctions are also excellent pathways for the flow of electrical current, which plays a critical role in coordinating the electrical activity in cardiac and smooth muscle.

EXHIBIT III

7,582,727 Pharmaceutical formulations of bivalirudin and processes of making the same

7,582,669 Aminoindazole derivatives as medicaments and pharmaceutical compositions including them

7,582,640 Vitronectin receptor antagonist derivatives, method for preparing same, use thereof as medicines and pharmaceutical compositions containing same

7,582,639 N-deacetylthiocolchicine derivatives, their use and pharmaceutical formulations containing them

7,582,628 Pyrrolo[3,4-c]pyrazole derivatives active as kinase inhibitors, process for their preparation and pharmaceutical compositions comprising them

7,582,625 Pyrrolo[3,4-c]pyrazole derivatives active as kinase inhibitors, process for their preparation and pharmaceutical compositions comprising them

7,582,624 Benzodiazepine derivatives and pharmaceutical compositions containing them

7,579,469 Glucocorticoid mimetics, methods of making them, pharmaceutical compositions, and uses thereof

7,579,364 N-deacetylthiocolchicine derivatives, their use and pharmaceutical formulations containing them

7,579,341 Selected CGRP antagonists, processes for preparing them and their use as pharmaceutical compositions

7,579,336 Pharmaceutical composition comprising temozolomide ester

7,579,324 Conjugates of biologically active compounds, methods for their preparation and use, formulation and pharmaceutical applications thereof

7,579,321 Pharmaceutical compositions including low dosages of desmopressin

7,579,010 Botulinum toxin pharmaceutical compositions with a recombinant human serum albumin

7,576,241 Derivatives of amidines, their preparation, their use as medicaments and the pharmaceutical compositions containing them

7,576,219 Crystalline S-omeprazole strontium hydrate, method for preparing same, and pharmaceutical composition containing same

7,576,215 Quinolines and pharmaceutical compositions thereof

7,576,204 Heterocyclic macrolide pharmaceutical agent, a method of producing the same and use of the same

7,576,120 Azabicyclic compounds, a process for their preparation and pharmaceutical compositions containing them

7,576,099 Amide derivatives as ion-channel ligands and pharmaceutical compositions and methods of using the same

7,572,829 Pharmaceutical compositions useful in prevention and treatment of Beta-amyloid protein-induced disease

7,572,641 Pharmaceutical compositions comprising meloxicam and methods of their preparation

7,569,574 Purine derivatives, the preparation thereof and their use as pharmaceutical compositions

7,569,565 Pharmaceutical composition for the prevention or treatment of cerebral oedemas

EXHIBIT IV

Review

Anti-inflammatory effects of aspirin and sodium salicylate

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Abstract

Aspirin (acetylsalicylic acid) is one of the most widely used drugs worldwide. It acetylates cyclooxygenases thereby irreversibly blocking the conversion of arachidonic acid to prostanoids. Biotransformation of aspirin yields salicylate, a compound that possesses similar anti-inflammatory potency as aspirin but lacks aspirin's inhibitory effect on the activity of isolated cyclooxygenase. This article is aimed at providing an overview about the often conflicting results concerning the mechanisms of action of aspirin and sodium salicylate. At present, there is no common agreement about the extent to which salicylate contributes to aspirin's anti-inflammatory properties, as well as there is still no final conclusion reached about the mechanisms of action of sodium salicylate. Several possible sites of action of salicylate have been suggested: It has been shown that in intact cells—but not in purified enzyme preparations—, sodium salicylate inhibits prostanoid biosynthesis. This effect seems to be prevented in the presence of high concentrations of arachidonic acid, which has been shown to interfere with inhibition by salicylate of cyclooxygenase-2-mediated prostanoid formation *in vitro*. Other possible sites of action that are not directly related to cyclooxygenase inhibition have been suggested based on observations made *in vitro* using high concentrations of aspirin and sodium salicylate. These effects target intracellular signaling mechanisms such as kinases, including the mitogen activated protein-kinases (MAPK) cascade. With the exception of reported salicylate-induced activation of p38 MAPK, observed effects are usually inhibitory. This may be one reason for the observation that, downstream to kinases, inhibitory effects of salicylates have been observed on several nuclear transcription factors, such as nuclear transcription factor kappa B (NF- κ B) or activator protein 1 (AP-1). Several reports have also shown interference by salicylates with the expression of cyclooxygenase-2, which, depending on experimental models, can be observed as inhibitory but also stimulatory effects. Antioxidant properties of salicylates, adenosine release induced by sodium salicylate and aspirin-triggered lipoxin formation are additional mechanisms that may contribute to anti-inflammatory properties of aspirin and/or sodium salicylate. An additional focus of this review is the discussion of interactions between aspirin, sodium salicylate and other non-steroidal anti-inflammatory drugs (NSAIDs), which are of particular relevance in the gastro-intestinal and cardiovascular systems. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Non-steroidal anti-inflammatory drugs; Cyclooxygenase; Prostaglandins

1. General pharmacological properties

Aspirin (acetylsalicylic acid) is one of the most widely used drugs with an average yearly consumption of 30 g per person in industrialized countries (Roth and Calverley, 1994); in the United States alone, 35,000 kg aspirin is consumed daily (Jack, 1997). After oral administration of an analgesic dose, about 50% is de-acetylated to salicylate already during and immediately after absorption. Plasma half-life of aspirin is about 15 min, that of salicylate is

between 2 and 30 h depending on concentration. More than 80% of circulating salicylate is bound to plasma proteins (Needs and Brook, 1985). In anti-inflammatory therapy, plasma concentrations of salicylate range between 150 and 300 μ g/ml; reversible tinnitus develops at 200–450 μ g/ml, hyperventilation and acidosis at concentrations higher than 350 and 450 μ g/ml, respectively (Insel, 1996). Uncoupling of oxidative phosphorylation by salicylate leads to increased oxygen consumption and CO₂ production, and is one reason (in addition to central stimulation of respiration by salicylate) for hyperventilation (Insel, 1996). In addition, uncoupling of oxidative phosphorylation decreases intracellular ATP formation and, consequently, induces the release of adenosine into extracellular fluids (Cronstein et al., 1994), an effect that has been suggested to contribute to the anti-

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inflammatory actions of salicylate (see Section 5.3). Metabolism of salicylic acid occurs through glucuronide formation (to produce ester and ether glucuronides), or conjugation with glycine (to produce salicyluric acid). In addition, a small fraction is oxidized to 2,5-dihydroxybenzoic acid (gentisic acid, which is conjugated with glycine to form gentisuric acid), 2,3-dihydroxybenzoic acid, and 2,3,5-trihydroxybenzoic acid (Insel, 1996).

2. A remarkable history

In plants, salicylic acid is synthesized from *trans*-cinnamic acid by decarboxylation to benzoic acid and further 2-hydroxylation of benzoic acid (Leon et al., 1995). The widespread occurrence of salicylates in plants not only provided convenient sources for extraction (therapeutic effects of extracts of willow bark containing salicin have been known for almost 2000 years) but apparently also serves the well being of plants, where salicylic acid plays a central role in the activation of defense responses after pathogen attack (e.g. Chen and Klessig, 1991; Chen et al., 1995; Klessig et al., 2002). Therapeutic administration of sodium salicylate in rheumatic disease was common already 120 years ago (Stricker, 1876); aspirin, devoid of the unpleasant sweet taste of salicylate, was registered 1899. It took 72 years, until Vane (1971) could demonstrate “inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs”.

3. Cyclooxygenase-1 and cyclooxygenase-2

Inhibition of prostaglandin formation by aspirin-like drugs is achieved by inhibition of cyclooxygenase (also referred to as prostaglandin H synthase) that converts arachidonic acid to prostaglandin H₂, which, in turn, is metabolized by specific synthases or non-enzymatically to individual prostanoids. The cyclooxygenase enzyme is bifunctional, the (i) cyclooxygenase site catalyzing the oxygenation of arachidonic acid to prostaglandin G₂, which then is reduced to prostaglandin H₂ by the (ii) peroxidase activity of the same enzyme. After the demonstration of an inducible form of cyclooxygenase (Fu et al., 1990; Xie et al., 1991), it has become clear that there are at least two isoforms of this enzyme: cyclooxygenase-1, considered to be the constitutive isoform (Smith and DeWitt, 1996), and cyclooxygenase-2, which can be induced by a variety of stimuli and is the major isoform responsible for prostaglandin biosynthesis in inflamed tissue (Herschman, 1996; Smith and DeWitt, 1996). This concept is, however, complicated by the demonstration of constitutive expression of cyclooxygenase-2 in several tissues including CNS, kidney and possibly even arterial endothelial cells subjected to shear stress (Harris et al., 1994; Topper et al., 1996; Yamagata et al., 1993).

4. Cyclooxygenase inhibition by aspirin and salicylate

Aspirin causes irreversible inhibition of cyclooxygenase activity by acetylation of an essential serine at the active site of the enzyme (Roth et al., 1975; DeWitt et al., 1990), which interferes with binding of arachidonic acid at the cyclooxygenase active site. In intact cells, the inhibitory potency (IC₅₀) of aspirin is usually reported to be between 2 and 20 μ M, independent of substrate (arachidonic acid) concentration. Aspirin can be regarded as non-selective cyclooxygenase inhibitor, although it seems to possess a somewhat higher potency in cyclooxygenase-1 models. Since aspirin is rapidly deacetylated to salicylate, it has been assumed that anti-inflammatory effects of aspirin are largely mediated by salicylate (Higgs et al., 1987). This assumption receives support by experimental evidence that in vivo, salicylate and aspirin exhibit similar anti-inflammatory potencies (Preston et al., 1989; Smith et al., 1975; White et al., 1985; Whittle et al., 1980). Therefore, the question as to the mode of action of salicylate becomes central for the understanding of aspirin pharmacology.

Early studies showing only weak inhibition of cyclooxygenase by salicylate have raised doubts whether inhibition of prostaglandin biosynthesis can fully explain the anti-inflammatory activity of salicylate (Smith, 1975; Smith et al., 1975; Vargaftig, 1978a). In vivo studies that determined effects of salicylate on prostaglandin concentrations in inflammatory exudates (Higgs et al., 1987; Chiabrando et al., 1989) produced no conclusive results. Moreover, interpretation of studies using prostaglandin measurements in inflammatory exudates was complicated by the inherent problem to separate cause and effect: Attenuation of the inflammatory response may lead to reduced prostaglandin levels, as well as inhibition of prostaglandin biosynthesis will attenuate the acute inflammatory response.

In purified preparations of cyclooxygenase-1 or cyclooxygenase-2, sodium salicylate is inactive up to 6.25 mM (Mitchell et al., 1994). In intact cells, most investigators find salicylate to be a weak inhibitor (IC₅₀ usually > 1.5 mM) of cyclooxygenase-1 (e.g. Patrignani et al., 1997; Giuliano and Warner, 1999; Warner et al., 1999). Salicylate's potency as cyclooxygenase-2 inhibitor seems to depend largely on experimental conditions, most reported IC₅₀ values range between 30 μ M (Mitchell et al., 1997) and 1.5 mM (Patrignani et al., 1997). Intriguingly, reports suggesting moderate cyclooxygenase-1 selectivity of salicylate (Mitchell et al., 1994) contrast with other studies suggesting preferential cyclooxygenase-2 inhibition (e.g. Warner et al., 1999).

Taken as a whole, inhibition by sodium salicylate of prostaglandin biosynthesis requires intact cells, and its potency is very much dependent on experimental parameters. As a possible explanation for salicylate's ineffectiveness in inhibiting purified cyclooxygenase activity, it has been suggested that cooperative elements (e.g. proteins) present in intact cells are required to facilitate the binding

of salicylate and/or arachidonic acid to cyclooxygenase (Mitchell et al., 1997). However, it seems prudent to consider alternative possibilities for observed inhibition of prostaglandin biosynthesis by salicylate in intact cells [e.g. indirect effects of salicylate, or activity of biotransformation products of salicylate (Whitehouse and Graham, 1996; Hinz et al., 2000)].

To find explanations for the observed variation in apparent potencies of salicylate to inhibit prostaglandin biosynthesis, recent studies (Mitchell et al., 1997; Giuliano et al., 2001) investigated the effects of arachidonic acid on salicylate effects. The results of these studies showed that the potency of salicylate to inhibit cyclooxygenase-2-dependent prostaglandin formation is critically dependent on the concentration of exogenously added arachidonic acid. In cyclooxygenase-2-induced human pulmonary epithelial cells, salicylate effectively inhibited prostaglandin biosynthesis in the presence of arachidonic acid $\leq 10 \mu\text{M}$ with an IC_{50} of $31 \mu\text{M}$, which was increased to $>625 \mu\text{M}$ in the presence of $30 \mu\text{M}$ arachidonic acid (Mitchell et al., 1997). Based on these experimental observations, it seems possible that the potency of salicylate is also influenced by the amount of endogenous arachidonic acid available under different experimental conditions.

The impact of endogenous arachidonic acid liberated by inflammatory stimuli could provide an explanation for reported low potency of salicylate in cyclooxygenase-2 assays conducted in the absence of exogenous arachidonic acid, for example, in A23187-stimulated cyclooxygenase-2 expressing cell lines (Warner et al., 1999: salicylate IC_{50} $482 \mu\text{M}$; Giuliano and Warner, 1999: salicylate inactive $< 1 \text{ mM}$), or endotoxin-stimulated whole blood (Patrignani et al., 1997: salicylate IC_{50} $1481 \mu\text{M}$), or endotoxin-stimulated mononuclear cells (Amann et al., 2001: salicylate IC_{50} $> 3 \text{ mM}$). It seems reasonable to assume that the calcium ionophore A23187 released large amounts of endogenous arachidonic acid. Although a similar argument can be made for endotoxin stimulation, the general question arises whether a cyclooxygenase-inhibitor that is subject to marked competitive inhibition by endogenous arachidonic acid can exhibit anti-inflammatory activity in inflamed tissue with high phospholipase A_2 activity, or in other words, whether weak competitive cyclooxygenase-2 inhibition by salicylate can fully explain its anti-inflammatory effects *in vivo*.

5. Salicylate effects on intracellular signaling pathways and other non-cyclooxygenase effects

Before reviewing evidence for effects of aspirin and sodium salicylate that are not directly related to prostaglandin biosynthesis inhibition, the topic of “relevant” concentrations of these compounds in experimental conditions needs to be addressed. Anti-inflammatory therapy with high-dose aspirin results in plasma salicylate concentrations of $0.95\text{--}1.9 \text{ mM}$. Taking into account a plasma protein

binding of $80\text{--}90\%$, the concentration of free salicylate can be expected to be in the range of $250 \mu\text{M}$. It is, therefore, justified to discuss results obtained with millimolar concentrations of salicylate in experimental systems that are devoid of the full drug binding properties of plasma (most studies using incubation media containing $10\text{--}20\%$ serum). The major problem in resolving this question lies in the very limited data concerning bio-distribution (at the tissue and cellular level) of salicylate, that is, about the actual concentration of salicylate at the sites of action. There are reports suggesting accumulation of acidic non-steroidal anti-inflammatory drugs (NSAIDs) such as salicylate in acidic compartments within the body (Graf et al., 1975; Brune et al., 1980); salicylate, for example, has been shown to accumulate specifically in inflamed tissue (Brune, 1977; Rainsford et al., 1980). In addition, intracellular accumulation of salicylate in tissue and immune cells (Brune, 1977; Raghoebar et al., 1987) may affect its local concentration. Therefore, it seems questionable whether plasma salicylate concentrations found in patients are reliable definitions of “relevant” in an *in vitro* situation. In particular, for discussing possible mechanisms of action of salicylate, it seems reasonable to take into account also results obtained in *in vitro* studies with concentrations of salicylate exceeding those normally found in plasma of patients. Important information about clinical pharmacological actions of salicylate may be neglected by using a narrow definition of “relevant” concentrations.

There are a considerable number of studies showing interference by salicylates with intracellular signaling pathways; effects that typically become apparent in concentrations in the low millimolar range. Within the scope of this review, a brief overview of the main intracellular targets of salicylates will be given. For more detailed information, the reader is referred to the review by Tegeder et al. (2001).

5.1. Kinases and transcription factors

High concentrations of salicylates have been shown to interfere with kinases, including the mitogen activated protein-kinases (MAPK) cascade. With the exception of reported salicylate-induced activation of p38 MAPK (Schwenger et al., 1997, 1998), observed effects are usually inhibitory. This may be one reason for the observation that, downstream to kinases, inhibitory effects of salicylates have been observed on several nuclear transcription factors. Among transcription factors, the focus of research has been the interaction between salicylates and nuclear transcription factor kappa B (NF- κ B).

NF- κ B has been regarded as a key element in the response of cells to inflammatory stimuli. NF- κ B belongs to a group of homodimers and heterodimers of Rel/NF- κ B proteins that bind to DNA target sites, where they directly regulate gene transcription. In most cells, NF- κ B is present in the cytoplasm as an inactive complex bound to inhibitory

proteins (I κ B). Stimulus-induced activation of an inhibitor κ -B kinase (IKK) complex results in proteolysis of I κ B, and consecutive activation of NF- κ B. NF- κ B then translocates from the cytoplasm to the nucleus, where it binds to the κ B-sites in the promoter region of target genes and regulates their transcription. Targets include pro-inflammatory enzymes, cytokines, chemokines, and cell adhesion molecules. Inhibition of the NF- κ B pathway by aspirin and salicylate has been shown by Kopp and Ghosh (1994) and several subsequent studies (Bayon et al., 1999; Grilli et al., 1996; Pierce et al., 1996; Yin et al., 1998). In apparent contrast to the hypothesis of NF- κ B-mediated anti-inflammatory action of salicylate are studies suggesting that pharmacological actions of aspirin and salicylates are mediated by inhibiting CCAAT/enhancer-binding protein binding and transactivation rather than by interference with NF- κ B (Saunders et al., 2001).

Other transcription factors, which have been reported to be affected by salicylates (cf. Tegeder et al., 2001), include activator protein 1 (AP-1), a protein complex consisting of products of the *jun* and *fos* oncogene families. AP-1 is activated by several pro-inflammatory stimuli such as TNF- α , and regulates the expression of genes involved in the immune and inflammatory responses, overlapping in several instances with the target genes of NF- κ B.

However, it remains doubtful to which extent these effects contribute to the anti-inflammatory activity of salicylates. In fact, experiments conducted in mice deficient in p105 (the precursor of the p50 component of NF- κ B), aspirin and sodium salicylate retained their anti-inflammatory efficacy (Cronstein et al., 1999), while the effect of dexamethasone, which is known to inhibit the activation of NF- κ B, was abolished. In addition, Mitchell et al. (1997) showed inhibition of prostaglandin biosynthesis by salicylate to be clearly independent of inhibition of NF- κ B activation.

5.2. Effects on expression of cyclooxygenase-2

With regard to salicylate's possible interference with the induction of cyclooxygenase-2, there are conflicting results. Several studies have shown that salicylate can inhibit cyclooxygenase expression in isolated cells (e.g. Wu et al., 1991; Xu et al., 1999; Saunders et al., 2001). However, the majority of studies find no such inhibitory effects of salicylate on the expression of cyclooxygenase isoenzymes (O'Sullivan et al., 1993; Barrios-Rodiles et al., 1996; Fernandez de Arriba et al., 1999; Hinz et al., 2000).

In a recent study, we have observed that sodium salicylate, used in concentrations similar to those shown to interfere with nuclear translocation of the transcription factor NF- κ B (Grilli et al., 1996; Kopp and Ghosh, 1994; Yin et al., 1998), can enhance the expression of cyclooxygenase-2 protein in endotoxin-treated human peripheral blood mononuclear cells. Similar results could be obtained with aspirin, but not other NSAIDs (Amann et al., 2001).

Stimulation of cyclooxygenase-2 by NSAIDs has been reported previously (Meade et al., 1999; Paik et al., 2000). These effects were mediated through activation of peroxisome proliferator-activated receptors (PPARs), a mechanism that cannot account for the cyclooxygenase-2 increase in our experiments, since salicylates do not activate PPARs (Lehmann et al., 1997).

A possible explanation for a paradoxical stimulation of cyclooxygenase-2 by salicylates could be provided by the observation that salicylate can activate p38 MAPK (see Section 5.1), which, in turn, is required for transcription and stabilization of cyclooxygenase-2 mRNA (Guan et al., 1998; Dean et al., 1999). Other possible implications of the activation of p38 MAPK by salicylate have been discussed recently: Based on observations that p38 MAPK is essential for cytokine induction of inducible nitric oxide synthase (Faure et al., 1999; Guan et al., 1999; Clark et al., 2001) have discussed a possible causal relationship between salicylate's ability to enhance cytokine-induced expression of inducible nitric oxide synthase (Nishio and Watanabe, 1998; Durak et al., 1999; Shimpo et al., 2000), and the occurrence of paradoxical pro-inflammatory effects of salicylate in infectious disease of children.

5.3. Release of adenosine

The abovementioned work in p105 deficient mice (Cronstein et al., 1999) has provided results compatible with the view that a significant portion of the anti-inflammatory effect of salicylates is mediated through an adenosine-dependent mechanism. It has been suggested that uncoupling of oxidative phosphorylation by salicylates decreases intracellular ATP formation (Cronstein et al., 1994) and, consequently, induces the release of adenosine into extracellular fluids in sufficient quantities to exert anti-inflammatory effects.

5.4. Antioxidant properties

Sodium salicylate, serving as a chemical trap for hydroxyl radicals, the most damaging reactive oxygen species, has been shown to ameliorate hypoxia/reoxygenation injury in several tissues (van Jaarsveld et al., 1994; Colantoni et al., 1998). However, it remains at least doubtful, whether or not the low potencies of sodium salicylate or aspirin as radical scavengers (Ahnfelt-Ronne and Nielsen, 1987) have a bearing on their anti-inflammatory effects.

5.5. Aspirin-triggered lipoxins

Aspirin-triggered lipoxins are endogenous 15*R*-enantiomers of lipoxin A₄ and lipoxin B₄ with similar biological properties as native lipoxins (Gewirtz et al., 1998). Aspirin-induced acetylation of cyclooxygenase-2 prevents the formation of prostanoids, but, at the same time, allows cyclooxygenase-2-mediated formation of 15(*R*)-HETE

[= 15(*R*)-hydroxyeicosatetraenoic acid]. In cells expressing 5-lipoxygenase, 15(*R*)-HETE serves as substrate for the formation of 15-*epi*-lipoxin A₄, a compound that can exhibit anti-inflammatory properties primarily by down-regulating granulocyte activity (Serhan, 1997; Chiang et al., 2000). It seems important to note that, in contrast to aspirin, sodium salicylate does not promote the formation of lipoxins (Claria and Serhan, 1995).

This mechanism of aspirin-triggered lipoxin generation may be important particularly in inflamed tissue, where, in the presence of aspirin, cyclooxygenase-2 expressing endothelial cells can release 15(*R*)-HETE, which by a transcellular pathway is converted to 15-*epi* lipoxin A₄ in adherent 5-lipoxygenase expressing polymorphonuclear leukocytes (cf. McMahon et al., 2001). Since non-selective cyclooxygenase inhibitors as well as inhibitors of cyclooxygenase-2 will prevent the formation of 15(*R*)-HETE by acetylated cyclooxygenase-2 (Mancini et al., 1997), interference by these drugs with such aspirin action may be envisaged.

6. Interactions of aspirin, salicylate and other NSAIDs

There is a large body of literature concerning interactions between NSAIDs and aspirin and/or salicylate, of particular relevance being those reported in gastro-intestinal and cardiovascular systems.

6.1. Gastro-intestinal ulcerogenicity and gastroprotection

In experimental animals, sodium salicylate not only lacks ulcerogenicity (Glenn et al., 1979; Whittle et al., 1980), but can even prevent gastric mucosal damage induced by various stimuli such as indomethacin, aspirin, or ethanol (Ezer et al., 1976, 1984; Robert, 1981). There are, however, other noxious stimuli such as L-nitroarginine methylester (L-NAME) against which sodium salicylate is not protective demonstrating some specificity of the effect (Peskar et al., 2002a). Although sodium salicylate might act as a mild irritant on the gastric mucosa (Robert, 1981), this effect apparently is not causally related to gastroprotection: Inhibition of prostaglandin biosynthesis by indomethacin does not attenuate gastroprotection conferred by high doses of salicylate (Robert, 1981; Gretzer et al., 1998), while it effectively prevents gastroprotection induced by other mild irritants such as 20% ethanol.

Recently, however, it was found that threshold protective doses of sodium salicylate (15 mg/kg) were not only inhibited by the potassium channel blocker glibenclamide but also by indomethacin (Peskar et al., 2002b). These results suggest, that the protective effect of low concentrations of salicylate depends on the presence of endogenous prostaglandin, which in turn activates ATP-sensitive potassium channels. In contrast to salicylate, pretreatment with aspirin in doses up to 25 mg/kg did not inhibit ethanol-induced gastric mucosal damage (Peskar et al., 1988). Only

higher doses of aspirin (100–400 mg/kg) reduced the damaging effect of ethanol. This effect may be due to significant formation of salicylate from aspirin under these conditions (Peskar et al., 1988).

Interestingly, sodium salicylate exhibited extremely high potency in two experimental models of gastric mucosal damage (Peskar et al., 2002a), which seem to depend largely on cyclooxygenase-2 activity, that is, prevention of adaptive gastroprotection (Gretzer et al., 1998), and of ischaemia–reperfusion injury (Maricic et al., 1999). Selective inhibitors of cyclooxygenase-2 potentially abolish adaptive gastroprotection and significantly aggravate ischaemia–reperfusion injury (Gretzer et al., 1998; Maricic et al., 1999). In these models, sodium salicylate antagonized the effects of the selective cyclooxygenase-2 inhibitors at doses that were about three orders of magnitude lower than those preventing ethanol-induced gastric injury. So far it is not clear whether these salicylate effects result from a specific interaction with cyclooxygenase-2 inhibitors at the level of arachidonate metabolism or from functional antagonism (Peskar et al., 2002a). It has been observed previously (Trautmann et al., 1991) that direct gastroprotective effects of various non-steroidal anti-inflammatory drugs including several salicylates are not correlated with specific effects on gastric cyclooxygenase, 5-lipoxygenase or 15-lipoxygenase activity.

Although both adaptive gastroprotection (Gretzer et al., 1998) and ischaemia–reperfusion injury (Maricic et al., 1999) are sensitive to selective cyclooxygenase-2 inhibitors, only the latter experimental situation is characterized by induction and upregulation of cyclooxygenase-2 (Kishimoto et al., 1998). This effect can explain, why an inhibitor of cyclooxygenase-2 induction such as dexamethasone aggravates gastric ischaemia–reperfusion injury just as a selective cyclooxygenase-2 inhibitor, but does not affect adaptive gastroprotection (Peskar et al., 2002a). Since sodium salicylate in very low doses antagonizes the dexamethasone effect in ischaemia–reperfusion injury (Peskar et al., 2002a), it seems possible that salicylate interferes with dexamethasone at the transcriptional level for cyclooxygenase-2 induction. On the other hand, effects of salicylate unrelated to arachidonic acid metabolism cannot be completely excluded. Further investigations are necessary to clarify the pharmacological antagonism between dexamethasone and salicylate in ischaemia–reperfusion injury and possibly other pathological situations.

6.2. Cardiovascular system

While neither aspirin nor sodium salicylate in therapeutic doses have direct cardiovascular actions, larger doses depress the circulation both directly and by a central effect (Insel, 1996). It is well accepted that beneficial effects of aspirin in secondary prevention of myocardial infarction are primarily caused by irreversible inhibition of platelet cyclooxygenase-1 and the resulting inhibition of thromboxane A₂ formation. Therefore, particular attention has been focused at possible

interference with aspirin by other drugs that target cyclooxygenase.

Aspirin inhibits cyclooxygenase in platelets as well as in the vascular wall (Moncada and Vane, 1979). In contrast, sodium salicylate does not prevent thromboxane A₂ formation in platelet-rich plasma (Vargaftig, 1978a) and is a only weak inhibitor of thromboxane B₂ generation in clotting whole blood (Patrignani et al., 1997). Furthermore, sodium salicylate does not inhibit vessel wall cyclooxygenase (Whittle et al., 1980; Dejana et al., 1981). Therefore, it seems remarkable that sodium salicylate, being almost inactive as a direct inhibitor of cyclooxygenase-1, inhibits the effects of indomethacin and aspirin on thromboxane B₂ formation by platelets (Cerletti et al., 1981; Dahl et al., 1983).

These data demonstrate affinity of salicylate to the cyclooxygenase enzyme; drug interaction has been suggested to occur by binding of salicylate to a hypothetical supplementary binding side on platelet cyclooxygenase rather than directly on the substrate active side (Cerletti et al., 1981). In such a way, inhibition of generation of thromboxane B₂ and malondialdehyde by aspirin and indomethacin can be dose-dependently antagonized by sodium salicylate in vitro and in vivo (Vargaftig, 1978b; Ali and McDonald, 1979; Dejana et al., 1981; Merino et al., 1980; Cerletti et al., 1981; Dahl et al., 1983; Bucchi et al., 1986). A similar interaction between aspirin and sodium salicylate has been found on the cyclooxygenase of the vessel wall as determined by its major arachidonic acid-derived products, prostaglandin I₂ and 6-keto-prostaglandin F₁α, respectively (Dejana et al., 1981).

More detailed information is available about possible interference by NSAIDs with aspirin-induced inhibition of cyclooxygenase activity. For cyclooxygenase-1, it is known that aspirin blocks the access of arachidonic acid to the catalytic site by irreversibly acetylating a serine residue near the catalytic site. Prior occupancy of the catalytic site by competitive cyclooxygenase inhibitors therefore can prevent aspirin from gaining access to its binding site (Livio et al., 1982; Rao et al., 1983). A recent study has shown that such interaction is clinically important in patients receiving low-dose aspirin together with ibuprofen, which prevented aspirin-induced inhibition of serum thromboxane B₂ formation and platelet aggregation. A similar interference with cyclooxygenase-1 inhibition by aspirin was not observed with acetaminophen, diclofenac, or the cyclooxygenase-2 selective inhibitor rofecoxib (Catella-Lawson et al., 2001).

7. Summary and outlook

Aspirin, which has been in routine use for nearly a century, has unique properties as anti-inflammatory drug. It acetylates cyclooxygenase thereby irreversibly blocking the conversion of arachidonic acid to prostanoids. Biotransformation of aspirin yields salicylate, a compound that possesses similar anti-inflammatory potency as aspirin. There is hardly a substance in clinical use that has produced so divergent

experimental results as salicylate; there is still no common agreement about its mechanisms of action. Interest in the pharmacology of aspirin has received a boost by the developing awareness that the therapeutic potential of aspirin-like compounds seems much greater than envisaged only a few years ago.

Intense research efforts about the role of cyclooxygenase in tumor growth (cf. Gupta and Dubois, 2001; cf. Thun et al., 2002) or in CNS degenerative diseases (cf. O'Banion, 1999; cf. Hurley et al., 2002) are examples of emerging fields that may prove novel targets for aspirin-related compounds. In addition, pathophysiological concepts of atherosclerotic disease are shifting to the viewpoint that it constitutes basically a chronic inflammatory disease (Ross, 1999), a development that may also lead to re-evaluation of the role of aspirin in cardioprotection. Based on the observations of aspirin-resistant patients, it has been suggested that single nucleotide polymorphisms of cyclooxygenase-1 exist that could explain individual differences in aspirin sensitivity (Eikelboom et al., 2002). It has been reported that a search of publicly available records of single nucleotide polymorphisms within the human genome shows well over 100 single nucleotide polymorphisms within the loci of genes in the thromboxane A₂ synthesis pathway (Halushka and Halushka, 2002). It seems probable, therefore, that pharmacogenomics will provide intriguing results helping to understand individual variation of the response to aspirin and other NSAIDs and possibly also help to resolve open questions about their mode of action.

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